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Δψ and ΔpH are equivalent driving forces for proton transport through isolated F_0 complexes of ATP synthases

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ABSTRACT

The membrane-embedded F_0 part of ATP synthases is responsible for ion translocation during ATP synthesis and hydrolysis. Here, we describe an *in vitro* system for measuring proton fluxes through F_0 complexes by fluorescence changes of the entrapped fluorophore pyranine. Starting from purified enzyme, the F_0 part was incorporated unidirectionally into phospholipid vesicles. This allowed analysis of proton transport in either synthesis or hydrolysis direction with Δψ or ΔpH as driving forces. The system displayed a high signal-to-noise ratio and can be accurately quantified. In contrast to ATP synthesis in the *Escherichia coli* F_1F_0 holoenzyme, no significant difference was observed in the efficiency of ΔpH or Δψ as driving forces for H^+-transport through F_0. Transport rates showed linear dependency on the driving force. Proton transport in hydrolysis direction was about 2400 $H^+/(s\times F_0)$ at Δψ of 120 mV, which is approximately twice as fast as in synthesis direction. The chloroplast enzyme was faster and catalyzed H^+-transport at initial rates of 6300 $H^+/(s\times F_0)$ under similar conditions. The new method is an ideal tool for detailed kinetic investigations of the ion transport mechanism of ATP synthases from various organisms.

1. Introduction

F_1F_0 ATP synthases are complex molecular machines that reside in the inner membrane of mitochondria, the thylakoid membrane of chloroplasts or the cytoplasmic membrane of bacteria. They catalyze the synthesis of ATP from ADP and P_i with the energy stored in an electrochemical ion gradient across the membrane [1].

In fermenting bacteria, where respiratory enzymes are not active ATP synthases can work in reverse as ATPases to generate the membrane potential (Δψ) required for cell viability. Hence, both working modes of F_1F_0 ATP synthases are physiologically relevant.

The construction of the F_1F_0 ATP synthases is bipartite: The F_1 part with the subunits $\alpha_3\beta_3\gamma\delta\varepsilon$ harbors the catalytic sites for ATP synthesis and hydrolysis and protrudes into the aqueous compartment of the cell. The F_0 part in its simplest bacterial form consists of subunits ab_2c_{10-15}. It is membrane-embedded and catalyzes ion transport across the membrane. The number of c monomers in the oligomeric c-ring varies between species and concomitantly affects the ion/ATP ratio during ATP synthesis or hydrolysis [2].

ATP synthesis measurements with the purified enzymes of *Escherichia coli* and spinach chloroplasts have shown that electric potential and proton gradient are not equivalent driving forces [3]. It is unknown, however, if these discrepancies are F_0 intrinsic properties or if they are related to the holoenzyme.

To understand the principle of proton pumping in either direction and to investigate asymmetries between the synthesis and hydrolysis mode of F_1F_0 ATP synthases, a defined system is needed, in which unidirectional proton transport can be measured. In this work we developed a method to measure unidirectional proton transport through the F_0 part in synthesis and hydrolysis direction, energized selectively by either a pH difference (ΔpH) or an electric potential difference (Δψ) across the liposome membrane. The assay is based on the hydrophilic fluorophore pyranine which is entrapped inside proteoliposomes and allows proton transport to be quantified by monitoring fluorescence changes. The relative simplicity of the system accounts for a high reproducibility and straightforward determination of all parameters required for a quantification of H^+ transport rates. It is moreover generally applicable to ATP synthases from various origins and displays several advantages over described procedures.

2. Material and methods

Antibody against subunit b was a generous gift of Karl-Heinz Altendorf, University of Osnabrück, Germany. Chemicals were purchased from Fluka (Buchs, Switzerland) if not otherwise indicated.

Abbreviations: DCCD, dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TBT, tributlytin

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2.1. Enzyme purification and reconstitution into liposomes

For expression of the *E. coli* F_1F_0 ATPase plasmid pBWU13 [4] encoding the entire *atp* operon and an N-terminal His_{10}-tag on the β-subunit was used (ATGCACCATCACCATCACCATCACCATCACCAT was introduced by PCR in front of the starting ATG). Mutant plasmid containing the cAsp61Asn was generated by PCR using overlapping primers. The presence of the mutant codons was confirmed by automated sequencing of the cloned DNA at Microsynth AG (Balgach, Switzerland).

The plasmid was transformed and expressed in *E. coli* strain DK8 which lacks the entire ATP operon (ΔuncBEFHAGDC) [5]. Cells were grown overnight at 30 °C in LB medium supplemented with 100 μg/l ampicillin and 20 μg/l tetracyclin.

F_1F_0 ATPase was isolated and purified following a slightly modified procedure of Ishmukhametov et al. [6]. Briefly, 5 g cells were resuspended in 20 ml lysis buffer (200 mM Tris–Cl pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM K_2-EDTA and 2.5% glycerol) and subsequently passed twice through a French press cell at 100 MPa. Unbroken cells were removed by centrifugation for 5 min at 8000 *g*. Membranes were collected by centrifugation of the supernatant for 30 min at 200,000 *g*.

For solubilization, membranes were suspended in 10 ml extraction buffer (50 mM Tris–Cl pH 7.5, 100 mM KCl, 250 mM sucrose, 40 mM ε-aminocaproic acid, 15 mM *p*-aminobenzamidine, 5 mM $MgCl_2$, 0.1 mM K_2-EDTA, 0.2 mM DTT, 0.8% phosphatidylcholine, 1.5% octylglucopyranoside, 0.5% sodium deoxycholate, 0.5% sodium cholate, 2.5% glycerol, 30 mM imidazole) and gently stirred for 1 h at 4 °C. Unsolubilized material was removed by centrifugation for 45 min at 200,000 *g* and the supernatant passed through a 0.22 μm filter (Millipore AG, Zug, Switzerland). The filtrate was loaded on a Ni-IDA column (GE Healthcare, Glattbrugg, Switzerland) and washed with 10 column volumes of extraction buffer. The F_1F_0 ATPase was eluted with extraction buffer containing 400 mM imidazole. The elution fractions were analyzed on SDS-PAGE and stored in liquid nitrogen. Routinely, 5–10 mg purified ATP synthase was obtained per liter of culture.

Chloroplast F_1F_0 ATP synthase was isolated from spinach leaves as described [7]. Briefly, 1.5 to 2 kg of spinach leaves was homogenized in a sucrose-containing buffer with a blender. Subsequent filtration and centrifugation steps were performed to obtain a thylakoid membrane preparation (chlorophyll concentration of 5 mg/ml) and the samples were stored at −20 °C.

The ATP synthase was solubilized from the membranes, fractionated by ammonium sulphate precipitation and subsequent sucrose density gradient centrifugation as described [7]. The purified protein samples (5 mg protein/ml) were stored in liquid nitrogen.

2.2. Purification of *E. coli* F_1 complex

E. coli F_1 complex was purified as described previously [8].

2.3. Reconstitution of F_1F_0 ATP synthase from *E. coli* and chloroplasts

The F_1F_0 ATPase from *E. coli* and chloroplasts was reconstituted following a slightly modified procedure as described [6]. Soybean phosphatidylcholine (Sigma-Aldrich, Buchs, Switzerland) was dissolved at a concentration of 30 mg/ml in buffer A (10 mM Tricine–NaOH pH 8.0, 2.5 mM $MgCl_2$, 0.1 mM Na_2-EDTA, 0.2 mM DTT) and sonicated at 7.5 μ for 2×30 s on ice using a tip sonicator (Sanyo MSE Soniprep, München, Germany) to form unilamellar liposomes.

The suspension was adjusted to 1% sodium cholate from a 10% stock solution and mixed with F_1F_0 ATPase (lipid:protein ratio (w/w) 1:100). The mixture was incubated for 20 min on ice and subsequently 1 ml was loaded on a PD-10 column (GE Healthcare, Glattbrugg, Switzerland), preequilibrated with buffer A. Turbid fractions were pooled and the proteoliposomes collected by centrifugation for 45 min at 200,000 *g* at 4 °C.

2.4. Preparation of F_0 liposomes

F_1F_0 proteoliposomes prepared as described above were dialyzed overnight against 1000 volumes of stripping buffer (0.5 mM Tricine pH 8.5, 0.5 mM Na_2-EDTA) at 4 °C to remove the F_1 part. The sample was diluted with stripping buffer and F_0 liposomes were collected by centrifugation for 45 min at 200,000 *g* at 4 °C.

The liposomes were resuspended at 60 mg/ml in buffer B (2 mM MOPS–NaOH pH 7.2, 2.5 mM $MgCl_2$, 50 mM Na_2SO_4 or 50 mM K_2SO_4) including 1 mM pyranine and frozen in liquid nitrogen for 5 min, thawed in cold water and sonicated twice for 10 s in a water bath sonicator. The freeze/thaw/sonication procedure was repeated once.

To remove external pyranine, the liposomes were loaded on a PD-10 column equilibrated with buffer B containing either Na_2SO_4 or K_2SO_4. The turbid yellowish fraction was collected and concentrated by centrifugation (30 min at 200,000 *g* at 4 °C). The liposomes were resuspended at a lipid concentration of 120 mg/ml in the same buffer and stored at 4 °C. No loss in activity was observed within 7 days. Depending on the inner salt, they were denoted Na^+- or K^+-liposomes.

For equilibration at the desired pH, liposomes were diluted 20-fold in the respective buffer and incubated for 16–72 h at 4 °C.

2.5. Measurement of ATP hydrolysis activity

ATP hydrolysis measurements were performed using a coupled enzyme assay as described [9] with the following modifications. Instead of potassium phosphate, 50 mM Tris–Cl, pH 7.5 was used and Triton X-100 was omitted in the experiments with intact proteoliposomes.

2.6. Determination of buffer capacity of liposomes

The buffer capacity of the lipid headgroups was determined as described [10] with the following modification. Liposomes containing Na^+ or K^+ were prepared as described above in a buffer containing 50 mM Na_2SO_4, 2.5 mM $MgCl_2$ and 50 mM K_2SO_4, 2.5 mM $MgCl_2$, respectively, diluted to 10 mg lipid/ml and adjusted to pH 6 with 5 mM H_2SO_4. Aliquots of a 10 mM KOH solution were added and the pH change was recorded with a glass electrode. The buffering capacity at a certain pH value was calculated using the linear regression derived from three values in proximity of the desired pH (to obtain the amount of KOH used to change the pH value by one unit) and the weight of lipid used and was expressed as μM H^+/(g lipid × pH unit).

2.7. Proportion of empty and F_0-containing liposomes

The fraction of empty liposomes was determined as described [11]. Briefly, $\Delta\psi$-driven H^+-transport through F_0 was initiated by the addition of 8 nM valinomycin and followed until completion of the reaction. Then, 2 μl of a 2 mM stock solution of CCCP was added to allow H^+-transport into empty liposomes. The raw data were then corrected for the external pyranine and converted into pH traces as described in the manuscript. The pH change observed before CCCP addition (F_0 liposomes) was divided by the total pH change (F_0 and empty liposomes) to obtain the fraction of liposomes containing an F_0 part as lined out in Fig. 1D.

2.8. Fluorescence measurements

Pyranine fluorescence was measured with a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, USA). The excitation wavelengths were set to 405 and 460 nm and their emission at 510 nm was recorded with 2 Hz. The slits were set to 20 nm and the photomultiplier voltage was set to 550 V. During measurements, the fluorescence spectrophotometer can remain open, which facilitates *in situ* addition of chemicals.

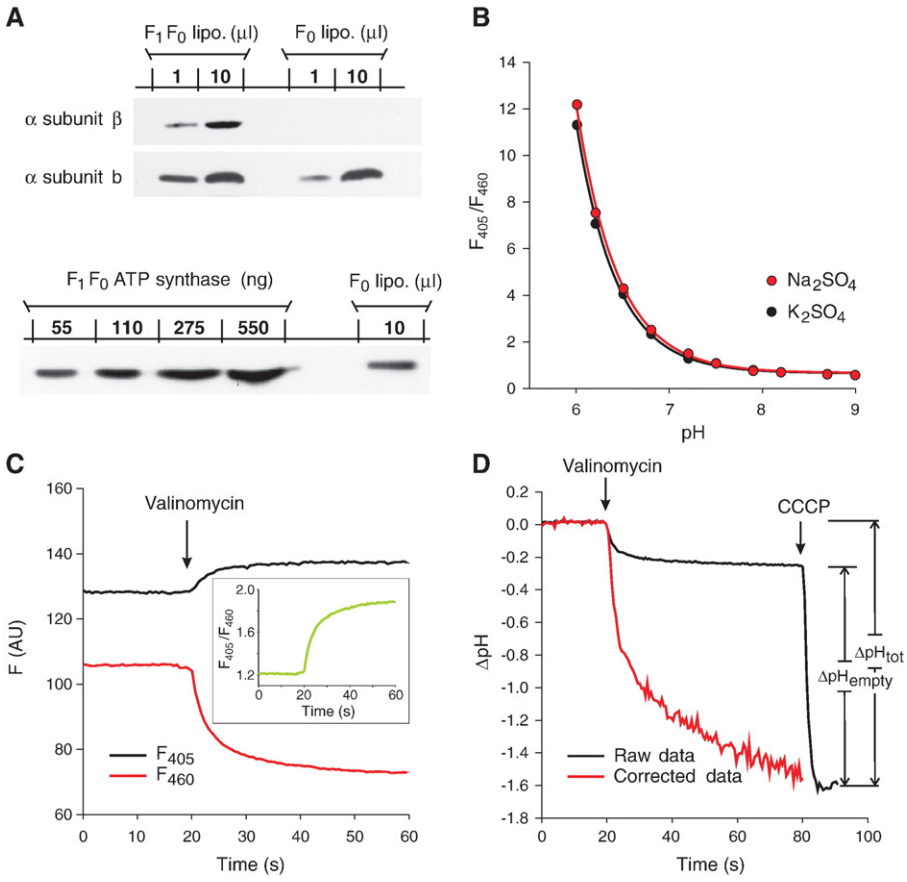

Fig. 1. (A) Western blot analysis of liposome preparations. (Upper panel) *E. coli* F_1F_0 ATP synthase was reconstituted into liposomes and the F_1 part was stripped off as described [6,23]. Samples of 1 µl and 10 µl before and after the stripping procedure were subjected to SDS-PAGE, blotted onto nitrocellulose and subunits β and b were detected by Western blotting. (Lower panel) A serial dilution of purified F_1F_0 ATP synthase of *E. coli* was used to quantify the amount of F_0 within the proteoliposomes. Indicated are the amounts of purified ATP synthase as calculated from protein determination and the amount of liposomes used per measurement. Samples were subjected to SDS-PAGE, blotted onto nitrocellulose and subunit b was detected by Western blotting. Western blot were scanned and signal intensities were obtained using QuantityOne (BioRad, Reinach, Switzerland) (B) pH dependence of pyranine fluorescence. Three microliters of empty liposomes (2 mM MOPS, pH 7.2, 2.5 mM $MgICl_2$, 1 mM pyranine and 50 mM Na_2SO_4/K_2SO_4) were prepared as described and mixed with 2 ml buffer at different pH values (2 mM buffer, 2.5 mM $MgICl_2$ and 50 mM Na_2SO_4/K_2SO_4). Equilibration of the pH values inside and outside of the liposomes was initiated by addition of CCCP (1.25 µM) and nigericin (0.25 µM in liposomes containing K_2SO_4) or monensin (0.25 µM in liposomes containing Na_2SO_4) and the fluorescence emission at 510 nm, excited at either 405 nm or 460 nm was recorded. The following chemicals were used to buffer at the indicated pH: MES (pH 6, 6.2, 6.5); MOPS (pH 6.8, 7.2); POPSO (pH 7.5, 7.9, 8.2, 8.4); CHES (pH 8.7, 9). The emission ratio F_{405}/F_{460} was then plotted against the pH values (circles) and a mathematical model ($y = a_0 - b * \ln(x_0 - x)$, whereby y and x correspond to the pH and the ratio F_{405}/F_{460}, respectively) was applied to fit the correlation as indicated (line). Shown are the mean values of two independent experiments. (C) Original data obtained in the measurements. In a 5 ml plastic cuvette, 5 µl to 10 µl of *E. coli* liposome solution (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 10 mM Na_2SO_4) was mixed with 2.5 ml of assay buffer (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 10 mM K_2SO_4) and florescence was recorded at 510 nm (excitation at 405 nm or 460 nm). After 20 to 30 s, H^+-transport was initiated by addition of 2 µl valinomycin from a 10 µM stock solution in ethanol and the sample was quickly mixed with a pipette. The ratio of the values (F_{405}/F_{460}) obtained were plotted against the time (inset). (D) Contribution of empty liposomes to the fluorescence signal. Using proteoliposomes containing F_0 part of spinach chloroplasts, a similar time course as described in 1C was recorded. After H^+-transport has reached a steady state level, 2 nM of CCCP was added to the sample and quickly mixed with a pipette to induce H^+-transport of empty liposomes. The trace was corrected for the external pyranine and converted into ΔpH values as described in the Materials and methods section. Assignments of total pH change (ΔpH_{tot}) and contribution of empty liposomes (ΔpH_{empty}) was used to calculate the amount of empty liposomes. The fluorescence values at F_{405} and F_{460} were then corrected by subtraction of the fluorescence contribution of empty liposomes and again the ratio was calculated and converted into ΔpH (red curve).

During kinetic measurements, the following series of manipulations was performed if not otherwise stated. In a 5 ml plastic cuvette, 2.5 ml of buffer was mixed with 10 µl of the preincubated proteoliposome suspension. A baseline was recorded for 20 to 30 s, before the H^+-transport was initiated by the addition of 2 µl of a 10 µM valinomycin stock solution in ethanol and rapid mixing with a pipette. This mixing procedure proofed to be more effective than using a magnetic stirrer during measurements. All transport measurements were performed at room temperature.

2.9. Determination of the inner liposome volume

The inner volume of a liposome suspension was determined using pyranine as membrane impermeable fluorophore. Samples (1 ml) containing liposomes (30 mg lipid/ml) in buffer C (buffer A supplemented with 50 mM KCl) were prepared in presence or absence of 1 mM pyranine (pyranine or control liposomes) as described above. The two liposome preparations were then diluted threefold with buffer C containing no or 1 mM pyranine to yield two suspensions containing the same total amount of pyranine. The liposomes were collected by centrifugation (200,000 *g*, 45 min, 4 °C) and resuspended in 1 ml of buffer C. The external pyranine was removed by gel filtration on a PD-10 column using buffer C. The turbid fractions were combined and the liposomes were collected by centrifugation (200,000 *g*, 45 min, 4 °C) and resuspended in 1 ml buffer C. The total amount of phospholipid was determined after centrifugation and thorough removal of excess of buffer by weighing the wet liposome fractions using a balance with an accuracy of 0.1 mg (Mettler Toledo, Greifensee, Switzerland). Typically, 30 mg of dry phospholipids displayed a total mass of 90–100 mg after hydration.

The total amount of pyranine in the two preparations was determined by fluorescence spectroscopy in buffer C supplied with 0.2% Triton X-100 (to solubilize the liposomes). Samples of 4 µl of either liposome preparation were mixed with 2.5 ml buffer C and the

fluorescence emission at 510 nm was measured (excitation wavelength was set to 412 nm). A standard curve using different amounts of pyranine (from a 10 μM stock solution) in buffer C with Triton X-100 was used to quantify the total amount of pyranine measured in the liposome samples. To determine the amount of incorporated pyranine, the fluorescence of the control liposomes was subtracted from the fluorescence signal of the pyranine liposomes. The inner volume was then calculated assuming an internal pyranine concentration of 1 mM.

2.10. Western blot analysis

Western blots using antibodies against subunit β (from rabbit, ABCam, Cambridge, UK) and against subunit b (from mouse, described in [12]) were used according to the protocol of the manufacturer. Secondary antibody incubation was performed with horseradish peroxidase conjugated to anti-rabbit and anti-mouse antibodies, respectively, and protein bands were visualized using the ECL detection system (GE Healthcare, Glattbrugg, Switzerland).

2.11. Mathematical fits

For the mathematical fits indicated, the program SigmaPlot (SyStat Software, Erkrath, Germany) was used.

3. Results and discussion

3.1. Unidirectional reconstitution

To analyze proton flux through the F_0 complex in a specific direction, proteoliposomes with a defined orientation of the F_0 complex are required. In a facile protocol for the reconstitution of *E. coli* ATP synthase, preformed liposomes are treated with Na^+-cholate and incubated with the purified enzyme. The detergent is removed subsequently by gel filtration [6].

The procedure was optimized for our purposes as described in Material and methods and the orientation of the reconstituted F_1F_0 ATP synthases was investigated by two independent methods. First, the enzyme orientation in the proteoliposomes was determined by measuring ATP hydrolysis activity. Since ATP is not membrane permeable, only the enzymes with the F_1 headpiece facing outwards contribute to ATP hydrolysis. After solubilization of the liposomes by a mild detergent, however, ATP is hydrolyzed by enzymes of either orientation. In a second procedure, the F_1 headpiece from outwards oriented enzymes was stripped off and removed by two subsequent washing steps with low ionic strength buffer containing EDTA. The efficiency of removing the external F_1 parts by this procedure was >98%. To determine the amount of inwards oriented enzymes, the liposomes were solubilized and ATP hydrolysis activity was determined. The results of both experiments (Table 1) show a unique orientation of the F_1F_0 ATP synthase, with >97% of the F_1 moieties facing outwards. These conclusions were confirmed by Western blot analyses with antibodies against subunit β and b of liposome preparations before and after stripping of the F_1 parts. The results of Fig. 1 show that the β subunit was completely removed by the stripping procedure while the amount of subunit b remained unchanged (Fig. 1A (upper and lower panel). From these experiments, an entirely uniform orientation of F_0 parts was inferred.

Table 1
Orientation of ATP synthase after reconstitution into liposomes

Additions	Activity (mU/mg lipid)	Rel. activity (%)	Rel. internal activity (%)
F_1F_0 liposomes			
ATP	11.67	31.37	
CCCP	36.17	97.19	
Triton X-100	37.21	100	2.81
F_0 liposomes			
ATP	0.40	1.08	
CCCP	0.61	1.64	
Triton X-100	1.32	3.56	1.92

Liposomes containing *E. coli* F_1F_0 ATP synthase were prepared as described in Material and methods. The specific ATPase activity was 18 U/mg protein. Half of the liposomes were treated with low ionic strength buffer as described to strip off external F_1 headpieces to form F_0 liposomes. A sample of 20 μl of liposomes was then mixed with 500 μl ATP hydrolysis assay buffer and the activity was monitored at 340 nm by a coupled spectrophotometric assay as described [9]. CCCP (2 μM final concentration) was added to prevent the formation of an inhibitory potential and Triton X-100 (0.1% final concentration) was added to solubilize the liposomes. The internal ATPase activity was calculated from the difference of activities before and after addition of Triton X-100.

3.2. Pyranine as indicator of internal pH change

Pyranine is a pH dependent fluorophore with high polarity due to three sulfonate groups. The resulting membrane impermeability makes it particularly suitable for entrapment into lipid vesicles (for review, see [13]). Using an emission wavelength of 510 nm, the fluorophore was excited at 405 nm or 460 nm and the ratio of the respective emission intensities at 510 nm was calculated. This ratio displays a very reproducible dependency on the pH value in the range from pH 6 to pH 9 (Fig. 1B). To measure pH changes inside liposomes, pyranine was entrapped into the F_0 proteoliposomes by two freeze/thaw/sonication cycles in the presence of salt (here: 50 mM K_2SO_4) to enhance fluorophore uptake. The external pyranine was subsequently removed by gel filtration and the liposomes were concentrated by centrifugation. This procedure does not significantly change the H^+-transport properties of the liposome preparation (data not shown, and [13]).

In initial H^+-transport experiments, the proteoliposomes were energized with a $\Delta\psi$ generated through a K^+/valinomycin diffusion potential and the emission traces at 510 nm excited at 405 nm or 460 nm respectively, were recorded (Fig. 1C). The course of the reaction is expressed as the ratio of the emission values between the two excitation wavelengths (Fig. 1C, inset).

The setup as described so far is sufficient for qualitative measurements in order to test the capability of a proteoliposome preparation to translocate protons. Within a single preparation of liposomes, the output is also quantitatively correct on a relative level (when no absolute transport rates are required). Effects of inhibitors or mutations can therefore be investigated at this level.

3.3. Measurement of the pH within proteoliposomes

For determination of absolute transport rates, the flux of protons between proteoliposomes and the environment had to be determined by measuring the pH within liposomes, while the external pH remained constant.

The fluorescence emission of liposomes at either wavelength stemed from pyranine in F_0-containing ($F_{in,p}$) and F_0-free liposomes ($F_{in,e}$) and from residual external pyranine (F_{ex}).

$$F_{tot} = F_{in,p} + F_{in,e} + F_{ex} \quad (1)$$

To determine the amount of external pyranine, the fluorescence of a liposome preparation (pH 7.2) in buffer with pH 7.2 was measured first, followed by a rapid shift of the external pH to ~6.8 after addition of 20 μl 50 mM sulfuric acid. This pH jump only affected fluorescence of the external pyranine while that of the internal pyranine remained unchanged. This allowed determination of the fraction of internal pyranine (x_{in}) in a liposome preparation as described in Appendix I:

$$x_{in} = \frac{F_{tot(405,pH\,6.8)} - F_{tot(460,pH\,6.8)} \cdot R_{pH\,6.8}}{F_{tot(405,pH\,7.2)} - F_{tot(460,pH\,7.2)} \cdot R_{pH\,6.8}} \quad (2)$$

The terms $F_{tot(460,\ pH\ 6.8)}$, $F_{tot(405,\ pH\ 6.8)}$ and $F_{tot(460,\ pH\ 7.2)}$, $F_{tot(405,\ pH\ 7.2)}$ correspond to the measured fluorescence values of a liposome

preparation (internal pH 7.2) in buffers with pH 6.8 and pH 7.2, respectively. The value for $R_{\mathrm{pH\ 6.8}}$, corresponding to the ratio of the fluorescence ratio F_{405}/F_{460} at pH 6.8, was obtained from Fig. 1B.

The absolute contribution of externally bound pyranine can be calculated with Eq. (3) for any given pH Y.

$$F_{\mathrm{ex(405,pH\ Y)}} = F_{\mathrm{tot(405,pHY)}} - F_{\mathrm{tot(405,pH\ 7.2)}} \cdot X_{\mathrm{in}} \tag{3}$$

$$F_{\mathrm{ex(460,pH\ Y)}} = \frac{F_{\mathrm{tot(405,pH\ Y)}} - F_{\mathrm{tot(405,pH\ 7.2)}} \cdot X_{\mathrm{in}}}{R_{\mathrm{pH\ Y}}} \tag{4}$$

The calculated amount of external fluorescence was subtracted from the measured fluorescence to obtain the total internal fluorescence at each wavelength. In addition, the internal fluorescence of F_0-free liposomes, whose internal pH does not change during the reaction was subtracted. The amount n_e of F_0-free liposomes was determined as described in the methods section. Values of 50–60% and 75–85% F_0-free liposomes for preparations from *E. coli* and spinach chloroplast, respectively were obtained (Fig. 1D). These considerations lead to the following time course for each wavelength.

$$F_{\mathrm{i(405)}}(t) = F_{\mathrm{tot(405)}}(t) - F_{\mathrm{ex(405)}}(t=0) - F_{\mathrm{tot(405)}}(t=0) \cdot n_e \tag{5}$$

$$F_{\mathrm{i(460)}}(t) = F_{\mathrm{tot(460)}}(t) - F_{\mathrm{ex(460)}}(t=0) - F_{\mathrm{tot(460)}}(t=0) \cdot n_e \tag{6}$$

The course of the reaction was expressed as function of time

$$r(t) = \frac{F_{\mathrm{i(405)}}(t)}{F_{\mathrm{i(460)}}(t)} \tag{7}$$

Using the mathematical fit from Fig. 1B, the ratio $r(t)$ can be converted into the corresponding pH(t) function.

For a solution containing 50 mM Na_2SO_4, the following term was obtained

$$\mathrm{pH}(t) = 7.17 - 0.523 \cdot \ln(r(t) - 0.372);\ R^2 = 0.998$$

For a solution containing 50 mM K_2SO_4, we obtained

$$\mathrm{pH}(t) = 7.21 - 0.509 \cdot \ln(r(t) - 0.368);\ R^2 = 0.997$$

3.4. Characterization of the experimental setup

A K^+/valinomycin diffusion potential was applied as driving force for H^+-transport. In these experiments, the concentration of valinomycin must be high enough to ensure that K^+-transport by valinomycin is not rate-limiting. Excessive valinomycin concentrations, however, which mediate H^+-transport must be avoided [14]. Therefore, a suitable valinomycin concentration has to be determined for each type of experiment. As depicted in Fig. 2A, samples with final

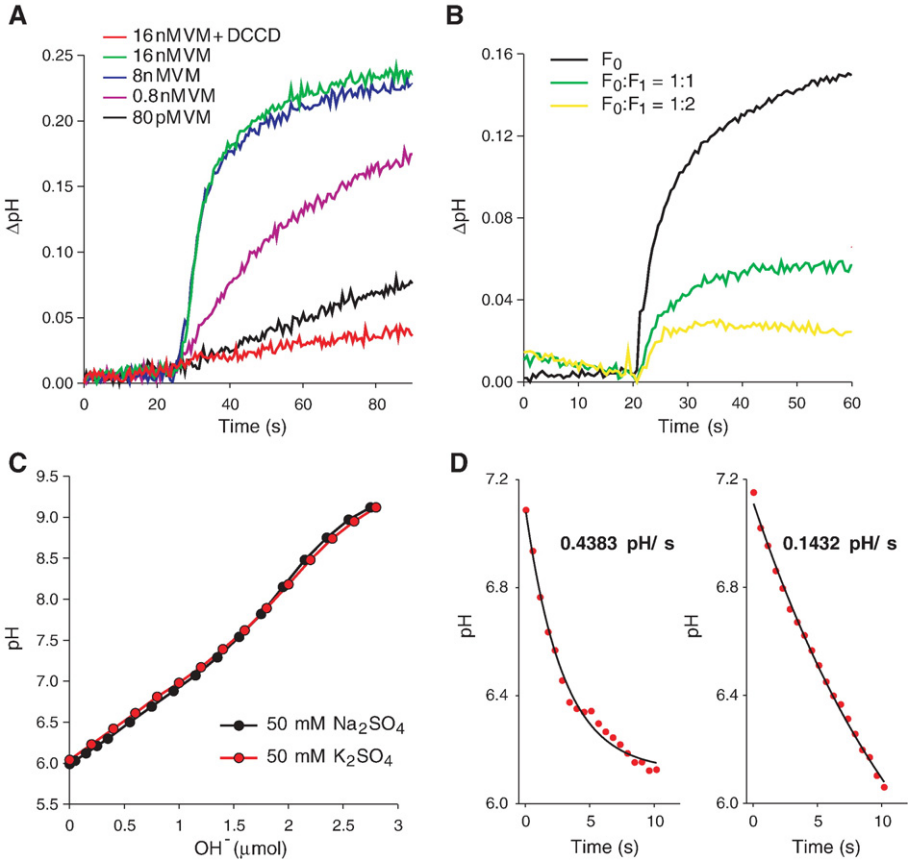

Fig. 2. (A) Effect of valinomycin concentration on initial transport rates. To test the effect of the valinomycin concentration, $\Delta\psi$ driven H^+-transport in synthesis direction was measured with the enzyme of *E. coli* (efflux from liposomes). Experimental conditions were similar as described in 1C, with the exception, that different amounts of valinomycin were added to initiate proton transport as indicated. As a negative control, DCCD-treated liposomes were mixed with the highest concentration of valinomycin (red trace). (B) Inhibition of F_0 H^+-transport after rebinding of soluble F_1. *E. coli* F_0 liposomes (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 50 mM Na_2SO_4, 0.125 mM K_2SO_4) were mixed with purified soluble F_1 part in the molar ratios indicated and incubated at 4 °C for 45 min. The liposomes were then diluted into K^+-assay buffer (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 50 mM Na_2SO_4, 0.125 mM K_2SO_4) and $\Delta\psi$-driven efflux was initiated by the addition of 8 nM valinomycin. (C) Determination of buffer capacity of liposome preparation. Liposomes prepared in either 50 mM Na_2SO_4, 2.5 mM $MgCl_2$ or 50 mM K_2SO_4, 2.5 mM $MgCl_2$ were diluted to 10 mg lipid/ml and adjusted to pH 6.0. Aliquots of a 10 mM KOH solution were added and the pH change was recorded with a glass electrode. Shown are the mean values of two independent experiments. (D) Determination of the initial rate of H^+-transport. Shown are experiments of $\Delta\psi$-driven H^+-transport in hydrolysis direction for the *E. coli* (left) and the spinach chloroplast (right) enzymes. The ten first seconds after valinomycin addition were used to fit the experimental data (red circles) mathematically (black curve, fitted as exponential decay with 3 parameters) and the initial rate was calculated from the first derivative.

valinomycin concentrations of 80 and 800 pM clearly showed a decreased uptake rate compared to samples with 8 and 16 nM valinomycin. On the other hand, only a minimal background H^+-transport was observed at 16 nM valinomycin when the ATP synthase was preincubated with 20 μM DCCD, a covalent inhibitor of the F_0 part. For further experiments a concentration of 8 nM valinomycin was used, if not otherwise indicated.

The integrity of the system was tested by incubating F_0 liposomes with isolated F_1 ATPase. After 45 min incubation at 4 °C, the liposomes were diluted into assay buffer and the sample energized with a $\Delta\psi$ and H^+-transport into the liposomes was determined (Fig. 2B). H^+-transport was drastically reduced (<10%) after incubation of the liposomes with isolated F_1, indicating that the F_1 parts had rebound to the membrane-embedded F_0 parts to prevent H^+-transport.

3.5. Initial rates of H^+-transport through F_0 of *E. coli* or spinach chloroplasts

Maximal rates for ATP synthesis and hydrolysis have been described to be around 50 to 250 ATP s^{-1} [4,15], corresponding to a maximal H^+-translocation rate of 150–1200 $H^+/(s\times enzyme)$. For the isolated F_0 part, however proton transport rates have been found that varied drastically up to very high values (70–100,000 $H^+/(s\times enzyme)$) [16–18], and only in recent years, comparable rates of 3000 to 8000 $H^+/(s\times enzyme)$ were obtained [11,19]. The experimental systems were quite complex and diverse and therefore, the data are difficult to compare. When the transport rate was measured in small particles of *Rhodobacter capsulatus*, the system was energized with flash induced H^+-transport by photosynthetic reaction centers and the relaxation of the induced potential was measured following the absorption changes of intrinsic chromophores [19]. To distinguish between H^+-transport through F_0 parts and other H^+-translocating proteins present in the system, the F_0 specific inhibitor oligomycin was used. The necessity of inhibitor use and primary proton pumps for energization can be omitted using reconstituted purified enzyme and an artificial energization by a K^+/valinomycin diffusion gradient. This has been applied to determine the proton transport rate through F_0 of the *E. coli* ATP synthase [11,16,17]. In these studies the pH change during H^+-transport was measured with a sensitive pH electrode in the external buffer medium [16,20]. Since the effective pH change is very small, a disadvantage of this method is a rather low signal-to-noise ratio.

In our experiments the disadvantages of contaminating enzymes or low signal-to-noise ratio were avoided by reconstituting the system from purified components and following the pH changes within the liposomes by a sensitive optical method.

In the following sections, the requirements for the determination of H^+-transport rates of the ATP synthases of *E. coli* and spinach chloroplasts are described.

3.5.1. Buffer capacity of the liposomes

The buffer capacity of the phospholipid headgroups lining the lumen of the liposomes exceeds by far the buffering capacity of the internal buffer (2 mM MOPS–NaOH) [10,13], but varies with the lipid composition. Therefore the buffering capacity of the employed liposome preparation was determined by titration with KOH as described in Material and methods (Fig. 2C). The buffering capacity was found to be approximately linear over pH segments of ~0.5 pH units and displays a value of ~100 μmol $H^+/(g$ soy bean lipid × pH unit) in the neutral range, which is in good agreement with similar experiments [10,13]. The absolute buffer capacity can be calculated from the amount of phospholipid used per measurement.

The contribution of the internal buffer to the buffer capacity is small (~1–5%), but can be calculated from the Henderson–Hasselbalch equation together with the internal volume of the liposomes. The internal volume was found to be 1.5 to 1.8 μl/mg lipid by pyranine fluorescence as described in Material and methods. Additionally, the liposome preparation was analyzed using electron microscopy (data not shown). The size distribution of the liposomes roughly ranged from 40 to 120 nm diameter which corresponds to an internal volume of $0.27–7.2\times10^{-18}$ l per liposome. If an average mass of 700 Da and an average surface area of 70 $Å^2$ is assumed for a single lipid molecule, a volume of 2–6 μl/mg lipid results. This estimation is slightly higher than the value obtained with the pyranine measurement but within the same range.

3.5.2. Number of functional F_0 molecules

A major difficulty in determining the transport rate per single F_0 complex is the determination of the number of active F_0 complexes in a measurement. Assuming that every liposome contains either one or no F_0 complex, the number of active F_0 complexes can be calculated from the total number of liposomes and the fraction of liposomes contributing to H^+-transport [11,19]. A weakness of this approach is the exact determination of the total number of liposomes which is very difficult. It can either be calculated from the inner volume of the liposomes [10] or the average liposome size (from electron microscopy or dynamic light scattering data) [11]. Thereby, the number is proportional to the volume of the liposomes. Since the volume is proportional to r^3, a little change in size determination drastically alters the number of liposomes. Alternatively, the amount of F_0 subunits in our preparation was quantified by Western blot analysis against subunit b. This allowed the calculation of the maximal amount of incorporated F_0 parts and the subsequent rate determination reflected a minimal value. Using a serial dilution of highly purified protein, an amount of 3.87 fmol of *E. coli* and 1.72 fmol of spinach chloroplast ATP synthase was incorporated per mg of lipid, respectively (Fig. 1A, lower panel). This reflected an overall reconstitution efficiency of ~13%.

3.5.3. Determination of initial rates

The experimental time courses were fitted mathematically to obtain the initial slopes as shown in Fig. 2D. From these data, the total number of translocated protons was calculated, taking into account the buffer capacity of the liposomes and the internal buffer. If the contribution of the internal buffer (~1–5%) was neglected, rates could alternatively be calculated without the knowledge of the internal volume. The maximal number of F_0 parts present in the reaction sample was calculated based on the amount of lipid per measurement. A typical calculation is exemplified in Appendix II. With these considerations, for the enzyme from *E. coli*, energized solely with a membrane potential of 120 mV, a minimal rate of H^+-transport of 2400 $H^+/(s\times F_0)$ part was obtained. Similarly, for the enzyme of spinach chloroplast, a minimal rate of 6300 $H^+/(s\times F_0)$ was obtained.

3.6. $\Delta\psi$ and ΔpH-driven H^+-transport in synthesis and hydrolysis direction

Proton uptake into unidirectional F_0 liposomes prepared by the method described above reflects the flux of protons in ATP hydrolysis direction. To measure the reverse flux of protons reflecting the ATP synthesis direction, a reverse $\Delta\psi$ (positive inside) was applied and the increase of the internal pH due to proton efflux was followed by the pyranine fluorescence technique. In addition, the effect of the two parameters of the proton motive force ΔpH and $\Delta\psi$ on the H^+ transport rate through F_0 was compared.

To prevent the formation of a counteracting membrane potential in experiments with ΔpH-driven H^+ transport, 50 mM K_2SO_4 and valinomycin were present on both sides of the liposomes. Conversely, liposomes energized by $\Delta\psi$ were incubated over night at the corresponding pH to assure that no ΔpH was present. To compare the effect of each driving force on the transport rates, a constant driving force of 73 mV, consisting either of a ΔpH = 1.2 or a $\Delta\psi$ (K^+_{in}/K^+_{out} ratio of 15.8) was applied. As control served an inactive mutant of the *E. coli*

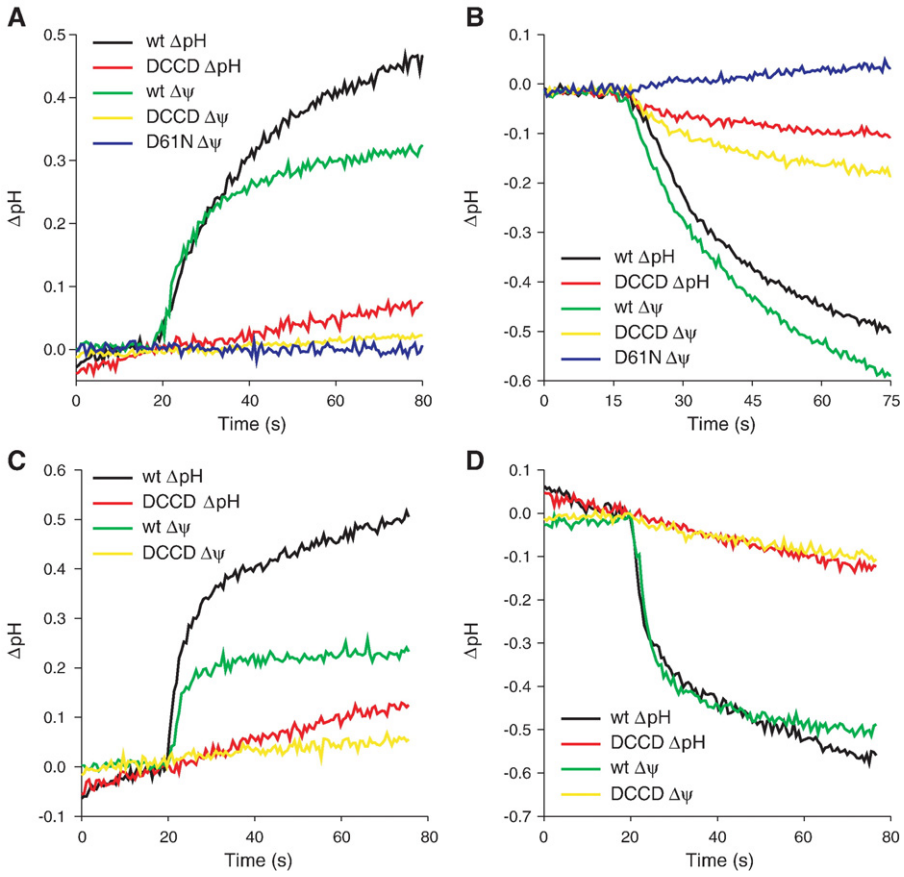

Fig. 3. (A) H^+-transport in synthesis direction in *E. coli* liposomes. In all measurements, a constant driving force of 73 mV in the form of either a Δψ or a ΔpH was applied. For Δψ-driven H^+-transport, 5 μl Na^+-liposomes (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 50 mM Na_2SO_4, 1 mM K_2SO_4) were mixed with 2.5 ml assay buffer (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 34 mM Na_2SO_4, 16 mM K_2SO_4) and H^+-transport was initiated with 8 nM valinomycin. For ΔpH-driven H^+-transport, 5 μl liposomes (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 50 mM K_2SO_4) were mixed with 2.5 ml assay buffer (2 mM POPSO–NaOH, pH 8.4, 2.5 mM $MgCl_2$, 50 mM K_2SO_4) and H^+-transport was initiated with 8 nM valinomycin. Initial rates resulting from these transport measurements were calculated as described in Fig. 2D and are summarized in Table 2. (B) H^+-transport in hydrolysis direction in *E. coli* liposomes. In all measurements, a constant driving force of 73 mV in form of either a Δψ or a ΔpH was applied. For Δψ-driven H^+-transport, 5 μl K^+-liposomes (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 50 mM K_2SO_4) were mixed with 2.5 ml assay buffer (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 46.875 mM Na_2SO_4, 3.125 mM K_2SO_4) and H^+-transport was initiated with 8 nM valinomycin. For ΔpH-driven H^+-transport, 5 μl liposomes (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 50 mM K_2SO_4) were mixed with 2.5 ml assay buffer (2 mM MES–NaOH, pH 6.0, 2.5 mM $MgCl_2$, 50 mM K_2SO_4) and H^+-transport was initiated with 8 nM valinomycin. Initial rates resulting from these transport measurements were calculated as described in Fig. 2D and are summarized in Table 2. (C) H^+-transport in synthesis direction in spinach chloroplast liposomes. As 3A, but liposomes containing spinach chloroplast ATP synthase were used. (D) H^+-transport in hydrolysis direction in spinach chloroplast liposomes. As 3B, but liposomes containing spinach chloroplast ATP synthase were used.

ATP synthase, where the essential cAsp61 was replaced by an asparagine [21]. When these liposomes were energized with Δψ, no H^+-translocation could be determined, reinforcing the tightness of the proteoliposome preparation. Curiously, when ΔpH was the sole driving force in these control liposomes, a slight drift of the signal was observed in the absence of valinomycin, indicating a slow H^+-translocation. The drift was similarly observed, when the F_0 part was labeled with the covalent inhibitor DCCD and therefore most likely reflects unspecific H^+-transport across the membrane.

The results of selected H^+ transport measurements are depicted in Fig. 3A–D. In synthesis direction the total amount (not the rate) of transported protons was bigger in the presence of ΔpH compared to Δψ, while both driving forces were comparable in hydrolysis direction. This effect is likely to be related to the mode of Δψ generation. Transport in synthesis direction requires an inside positive Δψ, which is obtained by a low internal and high external K^+ concentration. After valinomycin addition, K^+ ions flow into the liposomes and lead to a rapid rise of K^+ concentration due to the small internal volume of the liposomes. This again diminishes the Δψ and thus the driving force.

This effect is less pronounced, when an inverse Δψ is generated and a high K^+ concentration is present within the liposomes, since the external concentration can be assumed to remain constant.

For comparison of the different driving forces in each specific direction, the initial transport rates were calculated as described above and summarized in Table 2. For the enzyme from *E. coli*, H^+-transport in hydrolysis direction was found to be twice more efficient compared to the synthesis direction. Within one direction, the driving forces produced similar rates. The difference between hydrolysis and synthesis was less obvious in the spinach chloroplast enzyme, although slightly higher rates were obtained in the hydrolysis

Table 2
Initial H^+-transport through F_0

Organism	Driving force	Direction	$H^+/(s \times F_0)$	Reference
E. coli	Δψ = 108 mV	n. d.	3100	[11]
Bacillus PS3	Δψ = 94 mV	Hydrolysis	47 (at 25 °C)	[16]
Rhodobacter capsulatus	Δψ = 100 mV	Synthesis	6240	[19]
Chloroplast	Variable	Synthesis	Up to 100,000	[18]
E. coli	Δψ = 120 mV	Hydrolysis	2400	This study
Chloroplast	Δψ = 120 mV	Hydrolysis	6300	This study
E. coli	Δψ = 73 mV	Synthesis	640	This study
	Δψ = 73 mV	Hydrolysis	1350	This study
	ΔpH = 73 mV	Synthesis	560	This study
	ΔpH = 73 mV	Hydrolysis	1400	This study
Spinach chloroplasts	Δψ = 73 mV	Synthesis	2400	This study
	Δψ = 73 mV	Hydrolysis	3650	This study
	ΔpH = 73 mV	Synthesis	3100	This study
	ΔpH = 73 mV	Hydrolysis	3500	This study

Initial transport rates are summarized from Figs. 2D and 3A–D and from the literature. Shown are mean values of three to four measurements.

direction. Interestingly, ΔpH, which is the predominant driving force in chloroplasts, was found to be more efficient in driving proton transport in synthesis direction. Generally, rates at similar driving forces were up to three times higher in the chloroplast F_0 compared to the *E. coli* F_0 enzyme. This is in good corroboration with the maximal rates of 600 ATP s^{-1} and 240 ATP s^{-1} in the respective holoenzymes [15,22]. These findings further indicate a certain difference of the chloroplast and the *E. coli* enzyme in their efficiency to utilize the different driving forces. It is likely that these differences are even more prominent in the F_1F_0 holoenzyme.

3.7. Ohmic conductance of the E. coli F_0 part

In recent years, the F_0 parts of the ATP synthases of *Bacillus* PS3 (hydrolysis direction) and *R. capsulatus* (synthesis direction) were shown to exhibit a linear dependence of the H^+-transport rate on the driving force applied [16,19]. In contrary to the Na^+-translocating F_0 part of the ATP synthase of *Propionigenium modestum*, no voltage threshold was observed in the H^+-translocating enzymes [23]. We therefore utilized the described assay to investigate the kinetic properties of the *E. coli* F_0 part at pH 7.2. Na^+-liposomes (containing 50 mM Na_2SO_4, 0.5 mM K_2SO_4) and K^+-liposomes (containing 50 mM K_2SO_4) were prepared and used for a series of Δψ-driven efflux and influx measurements, respectively. The size of the membrane potential was modulated by varying the external K^+-concentration according to the Nernst equation. As depicted in Fig. 4A, linear relationships were obtained for both transport direction and no significant voltage thresholds were observed. As expected from the data in Fig. 3, transport in hydrolysis direction was roughly twice as efficient as in synthesis direction. A similar picture was obtained when ΔpH was the driving force for proton transport (Fig. 4B). In these experiments, K^+-liposomes (internal pH 7.2) were measured in K^+-containing buffers with pH values varying from pH 6 to 9, and H^+-transport was initiated with the addition of valinomycin. These data showed that Δψ and ΔpH are equivalent driving forces in the F_0 part of the *E. coli* ATP synthase. It remains elusive, whether the different findings in the F_1F_0 holoenzyme have to be attributed to the F_1 part or to altered F_0 properties after tight coupling with the F_1 part.

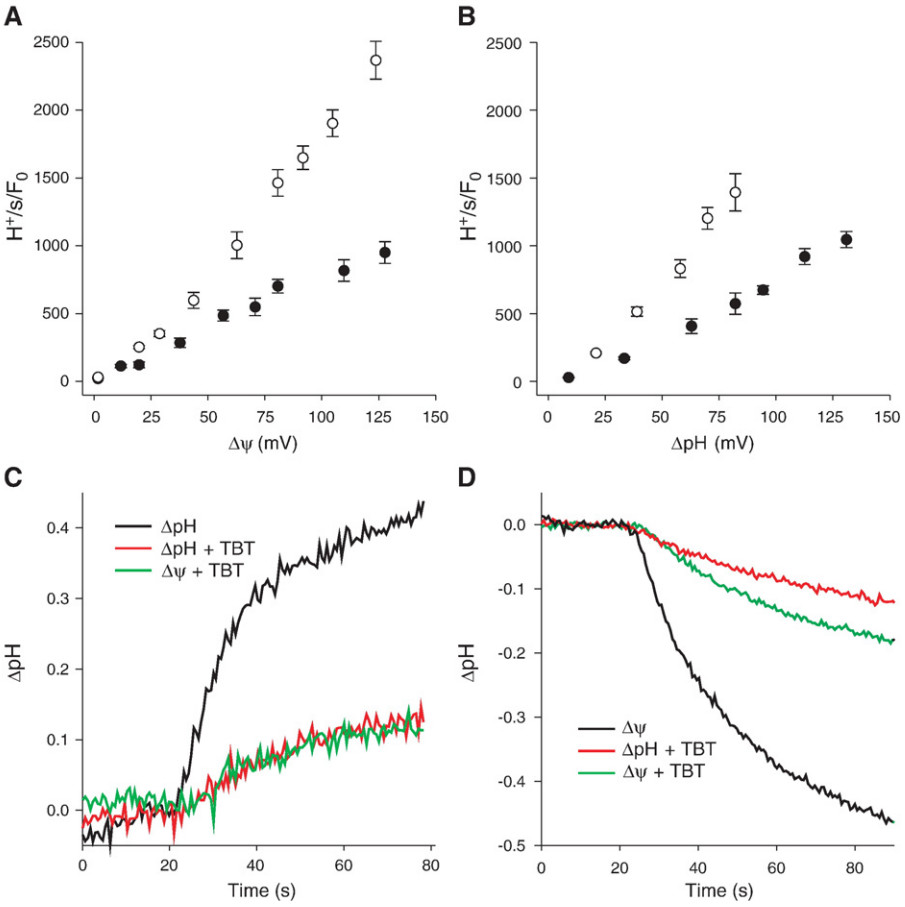

Fig. 4. (A) Effect of membrane potential on H^+-transport. Na^+-liposomes containing *E. coli* F_0 part (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 50 mM Na_2SO_4, 0.5 mM K_2SO_4) were used to measure Δψ-driven H^+-transport in synthesis direction (closed circles). The size of the membrane potential was calculated according to the Nernst equation and the K^+ concentration in the assay buffer was varied by mixing K^+-assay buffer (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 50 mM K_2SO_4) and Na^+-assay buffer (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 50 mM Na_2SO_4, 0.5 mM K_2SO_4) appropriately. Accordingly, Na^+-liposomes (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 50 mM K_2SO_4) were used to measure Δψ-driven H^+-transport in hydrolysis direction (open circles). Plotted are the initial rates as calculated in Fig. 2D. Shown are the mean values and the standard deviations of three measurements of two reconstitutions from the same enzyme preparation. (B) Effect of proton gradient on H^+-transport. K^+-liposomes containing *E. coli* F_0 part (2 mM MOPS–NaOH, pH 7.2, 2.5 mM $MgCl_2$, 50 mM K_2SO_4) were used to measure ΔpH-driven H^+-transport in synthesis (closed circles) and hydrolysis direction (open circles). The liposomes were mixed with assay buffer at different pH values and H^+-transport was initiated by addition of 8 nM valinomycin. The following chemicals were used to buffer at the desired pH values: MES (pH 6, 6.2, 6.5); MOPS (pH 6.8, 7.2); POPSO (pH 7.5, 7.9, 8.2, 8.4); CHES (pH 8.7, 9). Plotted are the initial rates as calculated in Fig. 2D. Shown are the mean values and the standard deviations of three measurements of two reconstitutions from the same enzyme preparation. (C) Inhibition of H^+-transport in synthesis direction by TBT. Shown are the results of the F_0 part of the spinach chloroplast ATP synthase. The F_0 part of *E. coli* produced similar results. An aliquot of 20 μl of empty liposomes (30 mg/ml in the respective assay buffer, containing no enzyme and no pyranine) was mixed with 2.5 ml of assay buffer and TBT was added to a final concentration of 500 nM. K^+-liposomes were then added as described and the baseline was recorded after 45 s incubation. H^+-transport was initiated with 1.2 nM valinomycin. (D) Inhibition of H^+-transport in hydrolysis direction by TBT. Shown are the results of the F_0 part of the *E. coli* ATP synthase. The F_0 part of spinach chloroplast produced similar results. The experiment was carried out as described in Fig. 4D, except that Na^+-liposomes were used for Δψ-driven H^+-transport in hydrolysis direction.

The unequal transport velocities in either synthesis or hydrolysis direction in the *E. coli* enzyme deserves attention. The finding emphasizes a functional asymmetry in the transport processes of the two directions. However, further experiments are required to elucidate, whether this phenomenon is due to different ion pathways or to unequal uptake/release affinities of the proton at either side of the enzyme at a certain pH value.

3.8. Inhibition of H^+-translocation by tributyltin chloride

TBT is a potent inhibitor of oxidative phosphorylation in general and ATP synthases in particular. While it has been shown to specifically bind to subunit a of the ATP synthase of *I. tartaricus* [24], no studies are reported about the interaction of TBT with purified F_0 during H^+-translocation. Therefore the ability of TBT to inhibit H^+-transport through the F_0 part of *E. coli* and spinach chloroplasts was investigated.

When TBT was added (final concentration of 500 nM) to the proteoliposomes in the assay buffer and incubated for 45 s, before the reaction was started with valinomycin, almost no inhibition was observed. The experiment was repeated with a DCCD-inhibited enzyme, and surprisingly, TBT stimulated a pH change indicative of an unknown transport, which is not related to the F_0 part. At elevated concentrations, TBT is also known to act as an anion/hydroxide exchanger [25]. Since only very little lipid was present in our measurement, accumulation of TBT in the liposome is likely to exceed this critical concentration and transport of hydroxide anions might generate the observed pH change. On the other hand it is possible that a certain TBT concentration is necessary to inhibit the ATP synthase (an apparent K_i of 200 nM has been determined for the *E. coli* enzyme in native membranes [24]). Therefore, a large excess of empty liposomes containing no pyranine was added to the sample to prevent accumulation of TBT in the F_0 liposomes and furthermore a decreased valinomycin concentration (1.2 nM) was used to prevent unspecific transport. Sets of inhibition experiments with the enzymes of spinach chloroplast and *E. coli* are shown in Fig. 4C and D, respectively. A significant decrease of H^+-transport was obtained in the presence of 500 nM TBT, irrespective of transport direction or driving force. It has to be considered that transport rates in the untreated enzyme are decreased due to the rate-limiting effect of valinomycin as shown in Fig. 1C and therefore the effective extent of TBT inhibition is even more pronounced.

4. Concluding remarks

Here we describe a reliable system for determination of H^+-transport through F_0 parts from various organisms. The formation of F_0 liposomes from purified components eliminates the possibility of unspecified ion fluxes through contaminating protein components. The reconstitution procedure yields a homogeneous orientation of the enzyme in the liposome bilayers with the cytoplasmic part of the enzyme facing outwards. The proteoliposomes were very tight towards unspecific proton diffusion or compensating charge transport.

To monitor pH changes during H^+-transport, pyranine was entrapped within the proteoliposomes. Pyranine is very hydrophilic and no leakage from entrapped molecules was observed. When measured ratiometrically, the fluorescence changes showed a very good correlation with the H^+ concentration in the range from pH 6 to pH 9. These properties make it a largely superior probe compared to ACMA, which is hardly quantitative and only allows measurement of an intravesicular pH decrease, but not pH increase. An additional advantage of pyranine is its relative ease of quantification, if the external pyranine is calculated and subtracted. Unlike to the measurement of pH changes in the external medium, internal pyranine measurements display a very high signal-to-noise ratio, which allows economic use of proteoliposomes for measurements.

The described system allowed for the first time to monitor and quantify H^+-transport through the ATPase F_0 part, under clearly defined conditions in both transport directions with each driving force. Controls with DCCD-inhibited or inactive enzyme displayed activities <5% and <3% respectively. Quantification of completely independent experiments (starting from different enzyme preparations) showed a good reproducibility (<12%) of the transport measurements.

In order to establish or to compensate for an electric membrane potential, valinomycin was present in all measurements. No significant unspecific H^+ transport by valinomycin as reported by others was observed [11] under the experimental conditions applied. Certain care has to be taken, when TBT is used as an inhibitor, since it exhibits anion/hydroxide exchanger properties, but the problem can be circumvented by addition of empty liposomes and usage of decreased valinomycin concentrations.

The advantages and properties of the described experimental setup makes it particularly suitable for an extensive investigation of the utilization of different driving forces by the F_0 part of ATP synthases from different origins, that is currently in progress in our laboratory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2008.06.008.

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